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Microvesicle Production after Trauma and Its Clinical Impact on Venothromboembolism

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14. ABSTRACT

Polytrauma is most often caused from explosive devices and accounts for about 65 percent of injuries to our military personnel. The patients who have polytrauma are at increased risk of developing either bleeding and/or a clot in their veins which cause a life-threatening event known as venous thromboembolism (VTE). We began enrollment of patients into the study on 2 February 2011. As of 1 October 2014, we have successfully enrolled and collected blood samples on 1139 patients and 89 healthy volunteers. We have thus far analyzed plasma samples of over 443 patients and 89 volunteers. In our preliminary analysis of thrombin generation and procoagulant microvesicle analysis, we have observed that thrombin generation is accelerated early after traumatic injury and there are greater numbers of procoagulant microvesicles noted after traumatic injury relative to healthy volunteers.

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Introduction: Venous thromboembolism (VTE) is a combat casualty adverse event reported to the Department of Defense. Rates of symptomatic and asymptomatic deep vein thrombosis (DVT) and pulmonary embolism (PE) in trauma patients are as high as 44 and 24%, respectively. The current guideline is that all major trauma patients receive VTE chemoprophylaxis. This practice exposes those not at risk for thrombosis to potentially serious bleeding and there are no adequate laboratory tests currently available to target anticoagulant prophylaxis to those that need it most. The **central hypothesis** of this proposal is that traumatic injury results in the release of procoagulant and pro-inflammatory factors found both in plasma and microvesicles (MVs) derived from blood cells and injured tissues. The specific Aims of this study are:

Aim 1:

- Identify cellular origins and quantitate procoagulant microvesicles (MVs) defined by cell specific markers in patients with acute traumatic injury.
- Determine the basis of differences in thrombin generation.

Aim 1 will be achieved through a prospective cohort study of patients with major trauma, estimate the distribution over time of procoagulant MVs concentration by cell of origin and thrombin generation.

Aim 2: Develop a predictive signature for a pre-thrombotic individual: thrombin generation concurrent with thrombogenic microvesicles.

Body: To complete Aim 1, a single center prospective case-cohort study was begun on February 2, 2011. This report is the 4th annual report (notification of award: October 2010). The annual reports which covered Tasks and Milestones achieved during funding years 1, 2 and 3 were submitted on October 2011, 2012, and 2013 respectively. This current report will cover the overall progress made during the funding of this study thus far.

Task 4: First 44 Months of Patient Accrual/Milestones:

Patient Enrollment: As of October 1, 2014 (during the first 44 months of study), 2234 acute trauma patients were screened for study enrollment, 1038 patients were excluded, and 1139 acute trauma patients and 89 healthy volunteers were consented and enrolled in this study. We are currently awaiting response from 57 mail-in consents sent to trauma patients who were discharged prior to obtaining consents. To date, 77 patients developed VTE within three months after trauma. This is 3 VTE patients lower than projected target number of 80 VTE patients needed to be enrolled assuming we were to finish the study as planned on December 2014. Because the proposed study required approval by DOD prior to patient enrollment, about 4 months lapsed between date of the award and patient study enrollment. A 12-month no-cost extension has been requested in order to give us an opportunity to enroll additional trauma patients to meet this goal and to utilize the funds to cover the cost of post-discharge surveys and data analyses by statataicians.

Laboratory Progress:

1. *Flow Cytometer Equipment Change*: As reported in our October 2012 Annual report, we had to switch from Canto-1 to Canto-2 flow cytometers to perform the microvesicle analysis. This decision was made after multiple attempts to fix Canto 1 equipment due to increased non-

specific signals evident while running patient plasma samples. This change led to an extra 3.5 months expended on re-running the patient samples on the new equipment, Canto 2. This step was taken after consulting with MV experts from within and outside Mayo Clinic. Additionally, we have identified a commercially available reference plasma, Cryocheck (Precision Biologic, Dartmouth N.S.), which was used with every carousel of patient samples to ensure that our technique for MV analysis was consistent. Between two experienced research technologists, the coeffcient of variation (CV) using our reference plasma has consistently been in the 13 – 15% range.

2. *Calibrated Automated Thrombinogram (CAT):* As reported in our October 2012 Annual report, we have added a variation of the CAT assay. In addition to the performance of standard "PPP" CAT assay (5 pM Tissue Factor/ 4 uM PCPS -phospholipid), we performed, in parallel, "PRP" CAT assays using 1pM Tissue Factor only. The rationale for performing the PRP assays was to assess for endogenous phospholipid (PL) as vehicle for thrombin generation and test the hypothesis that trauma patients will have increased plasma procoagulant PL as a result of tissue injury. As with the MV analysis, Cryocheck was also used as reference plasma for every micro-titer plate of patient samples processed. Between two experienced research technologists, the Coeffcient of Variation (CV) has consistently been ≤ 9 %. We also observed significant lot-to-lot variation with the "PPP" reagent (Thrombinoscope BV, The Netherlands). See Appendix Figure 1 below. For example, we observed a 26% between-lot difference in thrombin peak height (nM). The data presented in the subsequent sections of this report are adjusted for lot to lot variations.

3. Demographic data: The mean ± SD Age (years) and injury severity score of trauma patients were 48.0 ± 20.9 and 14.5 ± 10.7, respectively; 69.7% patients were men and 12 patients died during their hospitalization for trauma. The trauma patients had the blood sampled at baseline, 6 hours, 12 hours, 24 hours, Day 3, day of VTE (if diagnosed) and on day of discharge (DC). Based on time of patient's presentation to our Trauma Center and logistical reasons such as earlier discharge, not all patients have had their blood drawn at all these time points.

4. *Laboratory Results:* CAT and MV analyses have been completed on plasma samples from 426 acute trauma patients and 89 healthy controls; we present preliminary statistical analyses on 415 patients who had CAT analysis performed of which 387 patients had platelet derived (CD42a), procoagulant (Annexin V positive) MV analysis performed. Data are presented as median and median (min, max) unless otherwise noted; P value ≤ 0.05 was considered statistically significant.

Legend:

CD42a+ and Annexin V Pos (Q2)- Presence of procoagulant (phospholipid binding), platelet derived MVs
Annexin V Pos (Q2 Plus Q4) – Presence of all procoagulant MVs, regardless of cellular origin
Lagtime – time to initial thrombin generation (minutes)
Peak Height – peak thrombin activity (nM)
ttPeak – time to reach peak thrombin activity (minutes)
PPP reagent - Final concentration of 5pM Tissue Factor (TF) and 4 uM PCPS (phospholipid)

PRP reagent - Final concentration of 1 pM TF

b) we present preliminary statistical analyses on the first 238 patients enrolled who had CAT and 174 patients who had platelet derived (CD42a), procoagulant (Annexin V positive) MV analyses performed . When available serial plasma samples were analyzed, we note that: Relative to time of discharge, trauma patients have greater number of procoagulant MVs, shortened LagTime and increased Peak thrombin activity (Tables 1 and 2) early after injury. Towards the end of discharge these values decrease:

Table 1	Baseline (n=59)	6 hr (n=115)	12 hr (n=126)	24 hr (n=100)	Day 3 (n=65)	Discharge (n=72)
CD42a+/Annexin V Pos MVs (#/uL)	39 (20-80)	22 (12-42)	18 (11-35)	19 (11-31)	25 (15-43)	59 (35-82)
Any Annexin V Pos MVs (#/uL)	783 (363-1580)	299 (159-651)	237 (139-578)	229 (147-569)	343 (202-870)	332 (183-747)

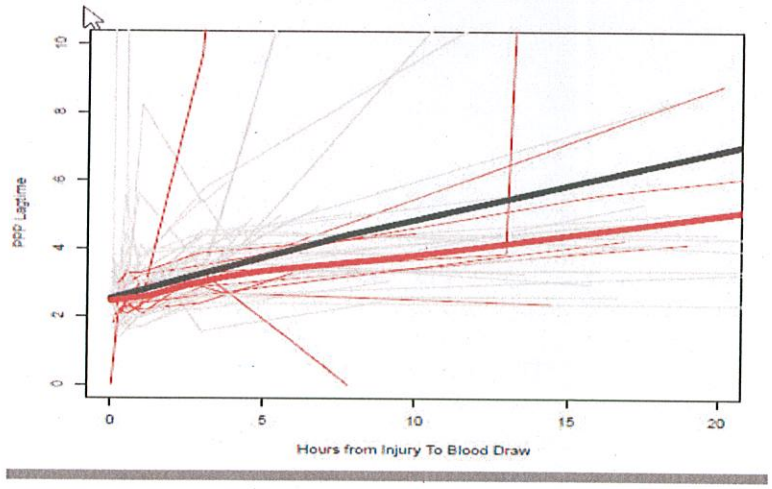
Table 2	Baseline (n=116)	6 hr (n=178)	12 hr (n=203)	24 hr (n=160)	Day 3 (n=108)	Discharge (n=120)
PPP LagTime (min)	3 (2.6-3.4)	2.9 (2.5 -3.3)	3.4 (2.6-3.4)	3.3 (2.7-3.7)	3.7 (3-4.3)	4.2 (3.4-6.0)
PRP LagTime (min)	7.3 (6.3-8.5)	7.7 (6.3-9.6)	8.7 (7.3-10.4)	9.2 (7.7-11.6)	10.6 (8.4 -13.1)	12 (9.2-19.8)
PPP Peak Height (nM)	244 (213-277)	227 (202-255)	222 (194-247)	207 (177-233)	237 (203-266)	225 (152-265)
PRP Peak Height (nM)	79 (61-114)	52 (41-68)	41 (33-59)	39 (28-53)	34 (27-51)	36 (14-42)
PPP ttPeak (min)	5.5 (5-6.4)	5.3 (4.9-6.1)	5.6 (4.9-6.5)	5.7 (5-6.6)	6.0 (5.3-7)	7.4 (6-12.1)
PRP ttPeak (min)	14.7 (12.3-17.2)	16.7 (14.5-19.7)	18.6 (16.0-22.0)	18.8 (16.3-21.9)	20.3 (17.5-23.9)	22.8 (18.5-32.1)

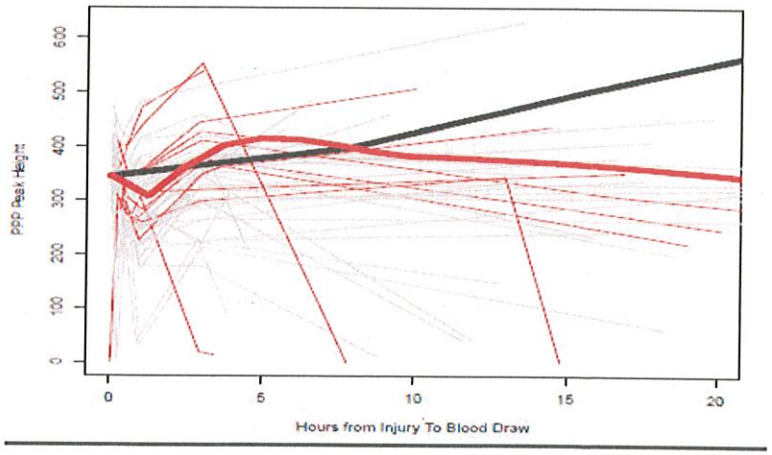
b) Trend Plot Over Time:

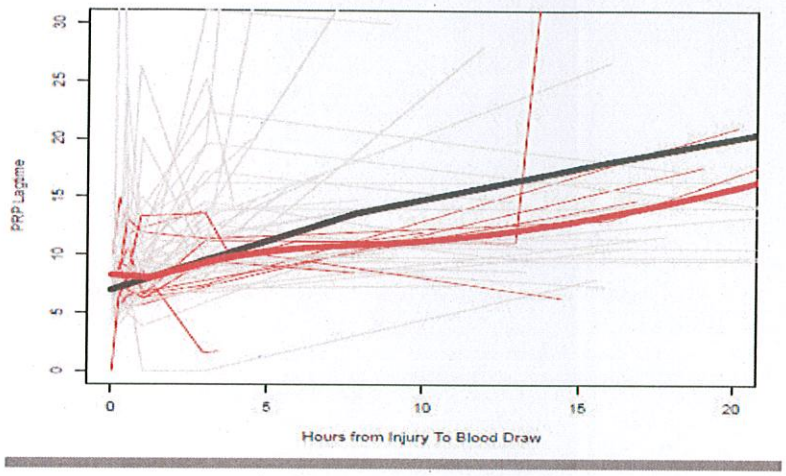
As we analyzed more plasma samples for CAT and MV analyses over the past 12 months, we analyzed data from 415 enrolled trauma patients. We initially ran trend plots on all CAT samples of all 415 patients (approximately 1900 samples); we then focused on CAT data samples from patients whose blood was drawn prior to any chemoprophylaxis based on in vitro heparin study done in our laboratory. Please refer to Appendix Figure 2 which shows that when unfractionated heparin, equivalent to the levels which would be found in blood of patients who receive chemoprophylaxis, was added to reference plasma samples during CAT analyses, thrombin generation was significantly impaired.

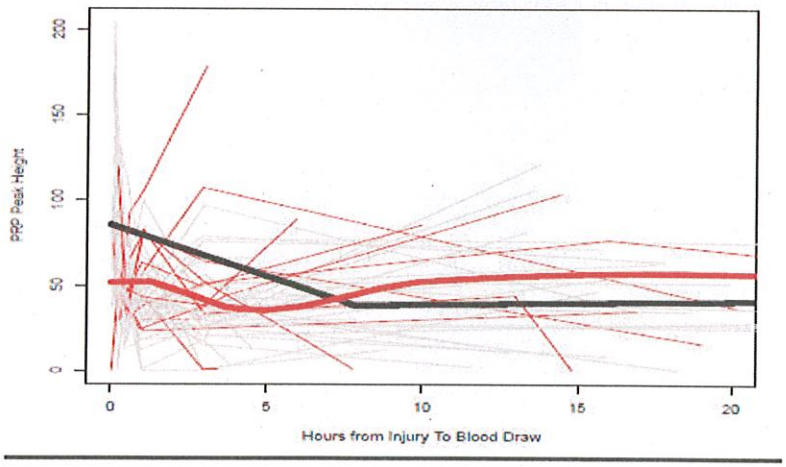
Hence, the following figures show trend plots of samples from patients prior to any heparin administration; with grey lines representing non-VTE patients, the red (thin) lines representing patients who developed VTE. The thick lines (black and red) represent a Loess smoother fit that show the overall trends in these patient groups (Non-VTE versus VTE, respectively). <u>The figures focus on the first 20 hour blood draw after injury as we had most data points during this time period.</u>

i) The following four trend plots pertain to CAT variables Lagtime and Peak Heights utilizing PPP and PRP reagents, respectively:

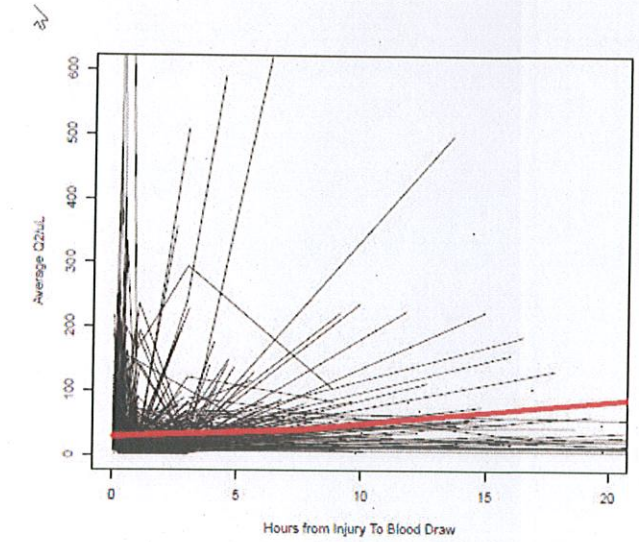


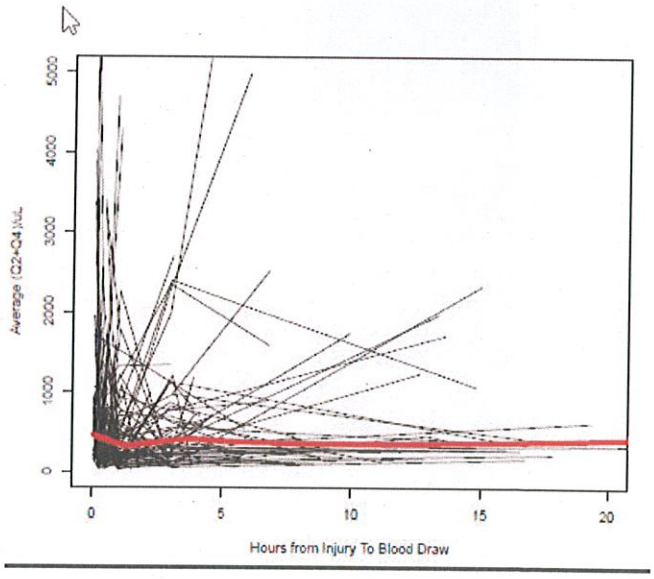



PRP Lagtime
0
5
10
15
20
25
30
0
5
10
15
20
Hours from Injury To Blood Draw


PRP Peak Height
0
50
100
150
200
0
5
10
15
20
Hours from Injury To Blood Draw

ii) The following two graphs depict platelet derived procoagulant (Average Q2) MVs counts per uL of plasma and All procoagulant MVs counts (Q2 Plus Q4) per uL of plamsa:





As evident in these trend plots over time, no overall specific pattern of CAT variables and MV counts exist during the early period (within 24 hours) after injury.

c) Further Analyses of CAT Variables:

i) Extreme CAT Value Analyses: Subsequent to tend plot analyses, we focused our attention on analyzing the extreme Lagtime, Peak Height and time to Peak CAT variables. Extreme values were defined as: lowest 5% Lagtime and ttPeak values and highest 5% Peak Height values. Such values would represent the most "hypercoagulable" CAT values:

Variable	N	Median (Min, Max)	Extreme 5% Value
PPP Lagtime (min)	1503	3.0 (0.0, 49.3)	2.0
PPP Peak Height (nM)	1503	326.2 (0.0, 659.1)	472.4
PPP ttPeak (min)	1503	5.0 (0.0, 297.8)	3.7
PRP Lagtime (min)	1504	8.9 (0.0, 67.9)	5.1
PRP Peak Height (nM)	1504	43.8 (0.0, 1916.2)	120.1
PRP ttPeak (min)	1504	18.1 (0.00, 1417.8)	10.9

ii) Also, the time (days) to reach extreme values relative to time of injury was examined. It appears that the extreme values are reached early after trauma:

Time to Extreme Values (days)	Median (Min, Max))
PPP Lagtime	0.31 (0.00, 19.81)
PPP Peak Height	0.24 (0.02, 36.84)
PPP ttPeak	0.31 (0.00, 19.32)
PRP Lagtime	0.43 (0.00, 19.81)
PRP Peak Height	0.19 (0.02, 16.77)
PRP ttPeak	0.33 (0.00, 19.81)

iii) We then looked for presence of associations between extreme CAT variables and relevant clinical variables (age, sex and injurity severity score):

PPP Extreme Lagtime

	OR (CI)	P-value
Cont. Age/5	1.11 (1.06, 1.16)	<0.001
Sex	1.61 (1.16, 2.23)	0.005
ISS	0.97 (0.96, 0.99)	<0.001

PPP Extreme Peak Height

	OR (CI)	P-value
Cont. Age/5	1.04 (0.99, 1.08)	0.11
Sex	2.06 (1.44, 2.94)	<0.001
ISS	1.02 (1.00, 1.03)	0.10

PPP Extreme ttPeak

	OR (CI)	P-value
Cont. Age/5	1.09 (1.04, 1.13)	<0.001
Sex	1.89 (1.36, 2.62)	<0.001
ISS	0.96 (0.95, 0.98)	<0.001

PRP Extreme Lagtime

	OR (CI)	P-value

Cont. Age/5	1.07 (1.03, 1.11)	0.002
Sex	2.17 (1.56, 3.03)	<0.001
ISS	0.98 (0.96, 0.99)	<0.001

PRP Extreme Peak Height

	OR (CI)	P-value
Cont. Age/5	1.02 (0.98, 1.07)	0.37
Sex	1.26 (0.87, 1.83)	0.22
ISS	0.99 (0.97, 1.00)	0.14

PRP Extreme ttPeak

	OR (CI)	P-value
Cont. Age/5	1.01 (0.97, 1.05)	0.78
Sex	2.13 (1.52, 2.98)	<0.001
ISS	0.97 (0.96, 0.98)	<0.001

iv) Receiver operating characteristic (ROC) curves were then generated to see how well CAT variables (from any non-heparinized blood samples collected during the first 8 hours after injury) improve the prediction of future development of VTE when added to a multivariable model with age, sex and ISS:

Variable(s) in Model	C-statistic	p- value
Age, ISS and Sex	0.692	0.042
+Plus PPP Lagtime	0.793	0.003
+ Plus PPP ttPeak	0.791	0.002
+Plus PRP Lagtime	0.753	0.006
+Plus PRP Peak Height	0.747	0.026
+ Plus PRP ttPeak	0.734	0.024
+Plus PPP Peak Height	0.710	0.090

Receiver operating characteristic (ROC) curves generated thus far reveals that, in conjuction with age, sex and ISS, the Lagtime and ttPeak (using the PPP reagent) best correctly predict future VTE with model concordance of about 80%.

d) Further Analyses of Microvesicle Data:
   a. Extreme MV Value Analyses:

Variable	N	Median Time (Days)To Extreme (Min, Max)	Extreme 5% Value
Procoagulant MV (per uL plasma)	1734	0.3 (0.05, 2.0)	> 2278

   b. Receiver operating characteristic (ROC) curves were then generated to see how well MV variables improve the prediction of future development of VTE when

added to a multivariable model with age, sex and ISS. We did not note any improvement of prediction of VTE when MV variable was added to the model.

Key Research Accomplishments:

- Standardization of methods to perform the MV analysis by flow cytometry and thrombin generation by calibrated automated thrombinogram (CAT).
- Screened 2234 trauma patients and enrolled 1139 patients and 89 volunteers into the proposed study.
- Plasma sample analysis of 443 patients performed each patient having an average of three samples for CAT and MV analysis.
- Through a prospective cohort study of patients with major trauma, we estimated the post-trauma procoagulant MV concentration and thrombin generation distribution over time.
- Patients with "Extreme" CAT variables consistenly have greater injury severity scores (ISS) and were older.
- Receiver operating characteristic (ROC) curves generated thus far reveals that, in conjuction with age, sex and ISS, the Lagtime and ttPeak (using the PPP reagent) best correctly predict future VTE with model concordance of about 80%.
- Enrolled 95% of trauma patients who developed VTE for eventual data analysis as outlined in Aim 2.
- Currently, two abstracts resulting from this study have been accepted for presentation: 1)2014 Eastern Association of Surgery of Trauma (oral presentation) and 2) 60th Annual International Society of Thrombosis and Haemostasis (poster presentation). For the 2015 Annual meeting of the Eastern Association of Surgery of Trauma, an abstract, which addresses our proposed Aim1, has recently been submitted. It is entitled, "Thrombin generation and procoagulant microparticle profiles after acute trauma: A prospective cohort study."
- Due to the funding of the grant, we have analyzed MV data and recently published our findings on the effect of transfusion on the levels of procoagulant MVs: Dhillon SK, Houck ML, Jenkins DH, Rosedahl JK, Harmsen WS, Halling TM, Park MS. Transfusion of stored red blood cells in trauma patients is not associated with increased procoagulant microparticles. Accepted in J Acute Care Surg. 2014; 77(5): 674-678.

Reportable Outcomes:

- In the evaluation of the trauma patients as a group, thrombin generation is accelerated and elevated early after traumatic injury relative to their time of discharge.
- In the evaluation of the trauma patients as a group, there is greater number of procoagulant MVs after traumatic injury relative to their time of discharge.
- Reference plasma should be used in conjuction with patient samples to assess consistency in technique and methods.
- Reference plasma should be used in conjuction with patient samples to assess for any lot-to-lot variability of reagents purchased commercially.

Conclusion: Over the past 44 months since the study inception, we successfully enrolled and analyzed plasma samples of trauma patients. We estimated the distribution over time of procoagulant MVs by cell origin and thrombin generation (Aim 1) and found that, overall, patients are in a hypercoagulable state relative to their time of discharge. As evident on the trend plots over time, no overall specific pattern of CAT variables and MV counts were observed during the first 24 hours after injury. However, for those patients who develop "Extreme" CAT variables do so early after injury and they have consistenly greater injury severity scores (ISS). Furthermore, when receiver operating characteristic (ROC) curves were generated to assess for how well CAT variables (from any non-heparinized blood samples collected during the first 8 hours after injury) correctly predict future development of VTE, Lagtime and ttPeak (using the PPP reagent) best correctly predict future VTE with model concordance of about 80%. Further statisitcal analyses are currently underway to include all laboratory variables collected during first 24 hours of injury in addition to other clinical data, present at time of admission, which may improve our ability to predict future development of VTE in the actuely injured.

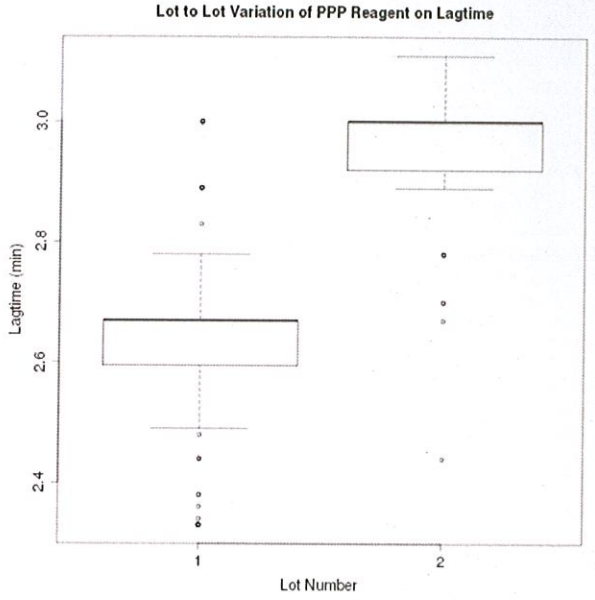
As outlined in Aim 2, the ultimate goal of our study to develop a VTE predictive signature model for an individual who, based on the thrombin generation and MV analysis, can be stratified into low versus high risk for deep vein thrombosis and pulmonary embolism after trauma. We have thus far enrolled 77 patients out of 80 VTE cases needed based on initial power analysis. As the power of the study is dependent on the number of VTE patients enrolled, efforts are currently underway to re-review the medical records of all enrolled patients within and outside Mayo Healthcare System to ensure that we have not missed any index cases. A 12-month no-cost extension has been requested in order to give us an opportunity to enroll additional trauma patients to meet this goal. Once desired enrollment is completed, we will assess the role of CAT and MV variables in predicting those trauma patients who subsequent to trauma, develop VTE. In thus doing, bleeding complications related to chemoprophylaxis could be minimized by assessing the blood physiology of individual patients rather than utilizing population-based algorithms. Conversely, it may be determined that other patients require more aggressive chemoprophylaxis than is currently administered.

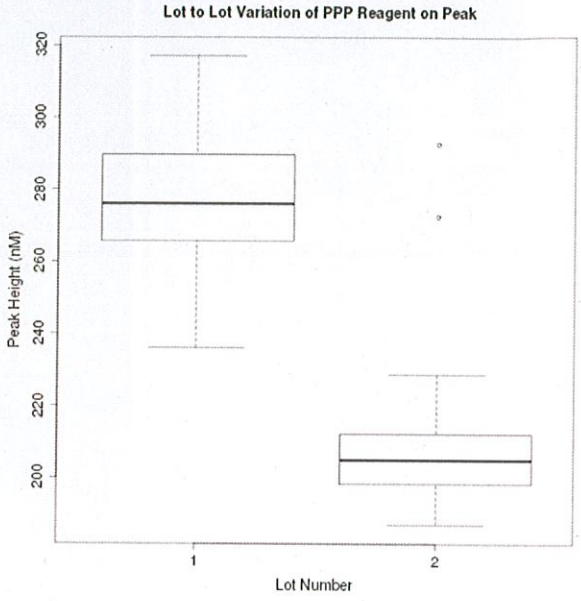
Appendix:

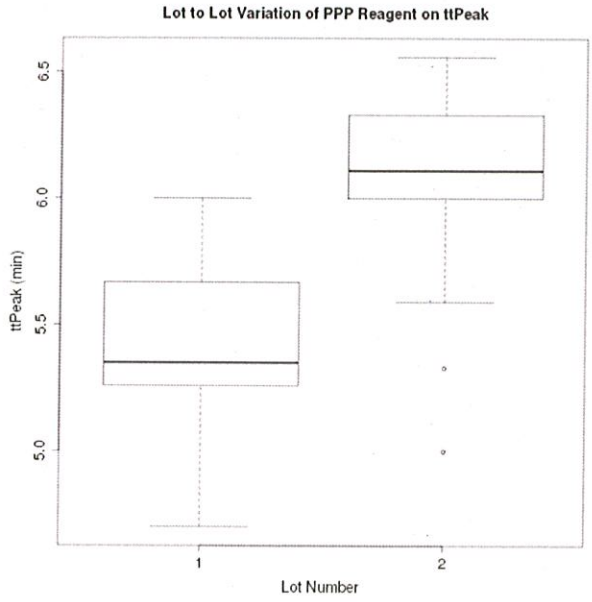
Figure 1:

a) Lot to lot variation of PPP reagents sold by Thrombinoscope BV accounted for during calculation of CAT parameters: Lag Time (min), Peak Height (nM) and ttPeak (min). The Graphs depicts the adjusted CAT results of Cryocheck reference plasma

- Lot-to-lot variation between lot #1 (original) and lot#2 (new)







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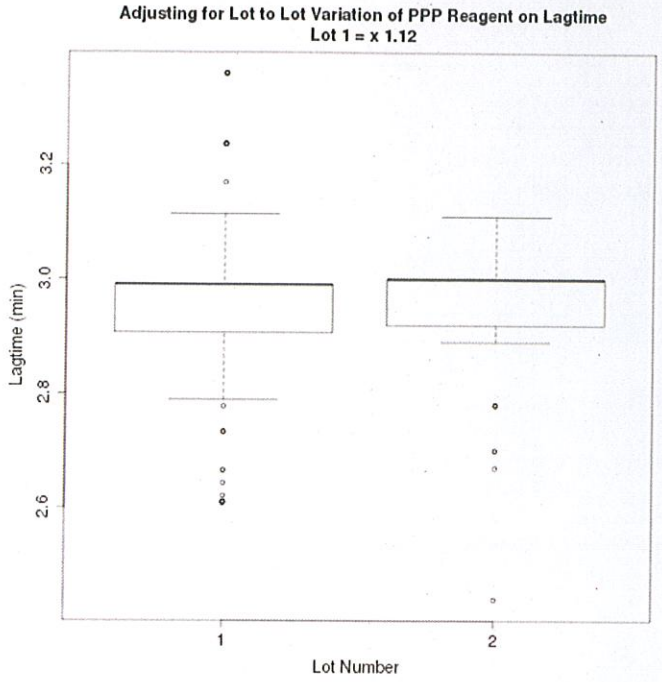

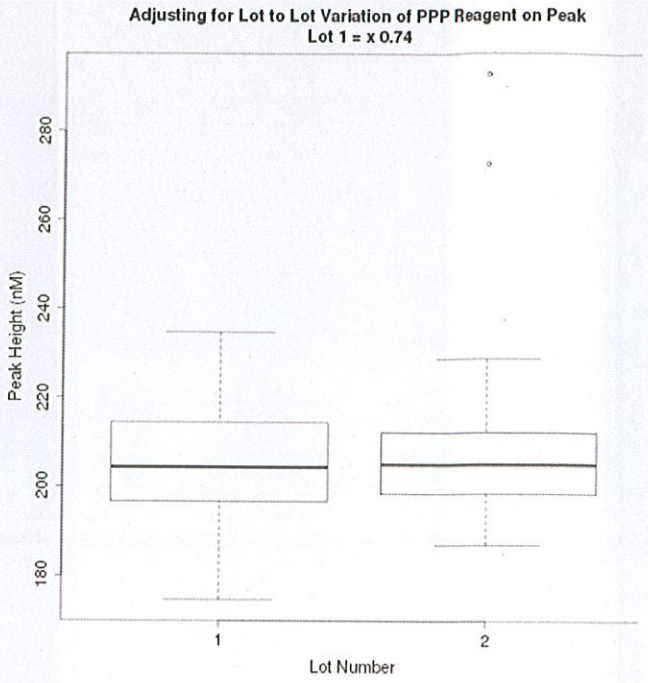

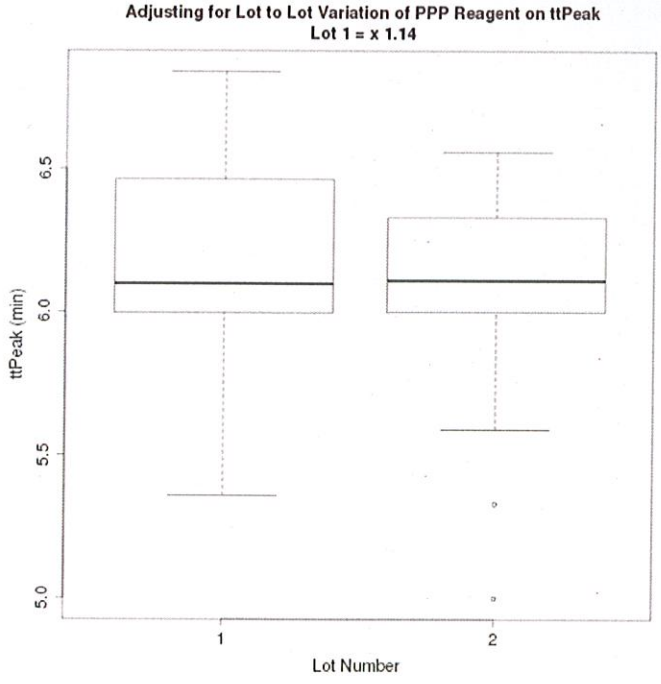

Figure 2: In order to assess the effects of prophylactic dose of heparin on CAT analysis, we performed CAT analysis of reference plasma (CC) with addition of 0.1unit and 0.2 unit per mL plasma concentration of unfractionated heparin to the microtiter wells. These values are equivalnet to what we expect to find in plasma of patients who receive prophylactic dose of heparin. We also ran the reference plasma (CC) without any heparin, as well as an additional high heparin dose of 0.4unit per mL plasma. We have found significant decrease in thrombin generation when heparin is added at all three doses: